Supplemental Data

Co-treatment with hepatocyte growth factor and TGF-β1 enhances migration of HaCaT cells through NADPH oxidase-dependent ROS generation

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Results

ROS generation is necessary for the enhancement of cell migration by combined growth factor treatment

To verify the signal transduction pathway responsible for the increase of re-epithelialization, wellknown inhibitors of various signaling pathways were pre-treated before the wound generation and cytokine treatment. Among the inhibitors tested, N-acetylcysteine (NAC), an antioxidant, along with PD98059 were the most effective ones in inhibiting re-epithelialization (Supplemental Data Figure S1), suggesting the involvement of ROS and ERK signaling in this process. Since ROS is widely recognized as one of the diverse intracellular signaling molecules when stimulated by growth factors, we next performed ROS analysis by using FACS and confocal microscope after staining with DCF-DA dye. FACS analysis revealed that ROS level slightly but statistically significantly increased after the scratch at both early (30 min) and late (20 h) time points when treated with both HGF and TGF-β1 at both 30 min and at 24 h compared with those without growth factor treatment (Figure 2A). Observation of DCF-DA fluorescence by confocal microscopy also revealed the increase of ROS by growth factor treatment. Notably, the increase of ROS was evident mainly at the margins of wound, suggesting that the scratch-induced damage to the cells was the source of initial signaling for ROS generation (Figure 3B, upper panels). To address whether the ROS generation is essential in HaCaT cell migration, N-acetylcysteine, an antioxidant, was treated prior to the application of growth factors and wound generation. Indeed, N-acetylcyteine significantly abolished cell migration in a dose-dependent manner (Figure 2B), suggesting the strong involvement of ROS in this process.

PI3K pathway is involved in re-epithelialization process, but not in ROS generation

Non-phagocytic cells produce superoxide anions in response to growth factors including PDGF and EGF (Sundaresan *et al*., 1995; Bae *et al*., 1997). Especially, ROS production in PDGF-stimulated cells has been shown to be mediated by sequential activation of phosphatidylinositol 3- kinase (PI3K), βPix, and Rac1, which then binds to Nox1 to stimulate its NADPH oxidase activity (Bae *et al*., 2000; Park *et al*., 2004). Therefore, we next investigated the involvement of PI3K pathway in the ROS generation and wound healing process. Interestingly, whereas HaCaT cell migration was inhibited in dose-dependent manners by either wortmannin or LY294002 (Figures 5A and 5B), wortmannin or LY294002 did not abolish the increase of ROS at both early (30 min; Figures 5C and 5D) and late time points (20 h; data not shown). These results suggest that PI3K pathway is indeed involved in HaCaT keratinocyte migration by growth factor co-treatment, but not through affecting ROS production. In fact, when we treated various signaling inhibitors prior to the addition of growth factors and wound generation, we observed that several different inhibitors could abolish the HaCaT cell migration (Supplemental Data Figure S1), which suggests that keratinocyte migration is a rather complex process which requires the har monious involvement of various intracellular signaling systems.

Discussion

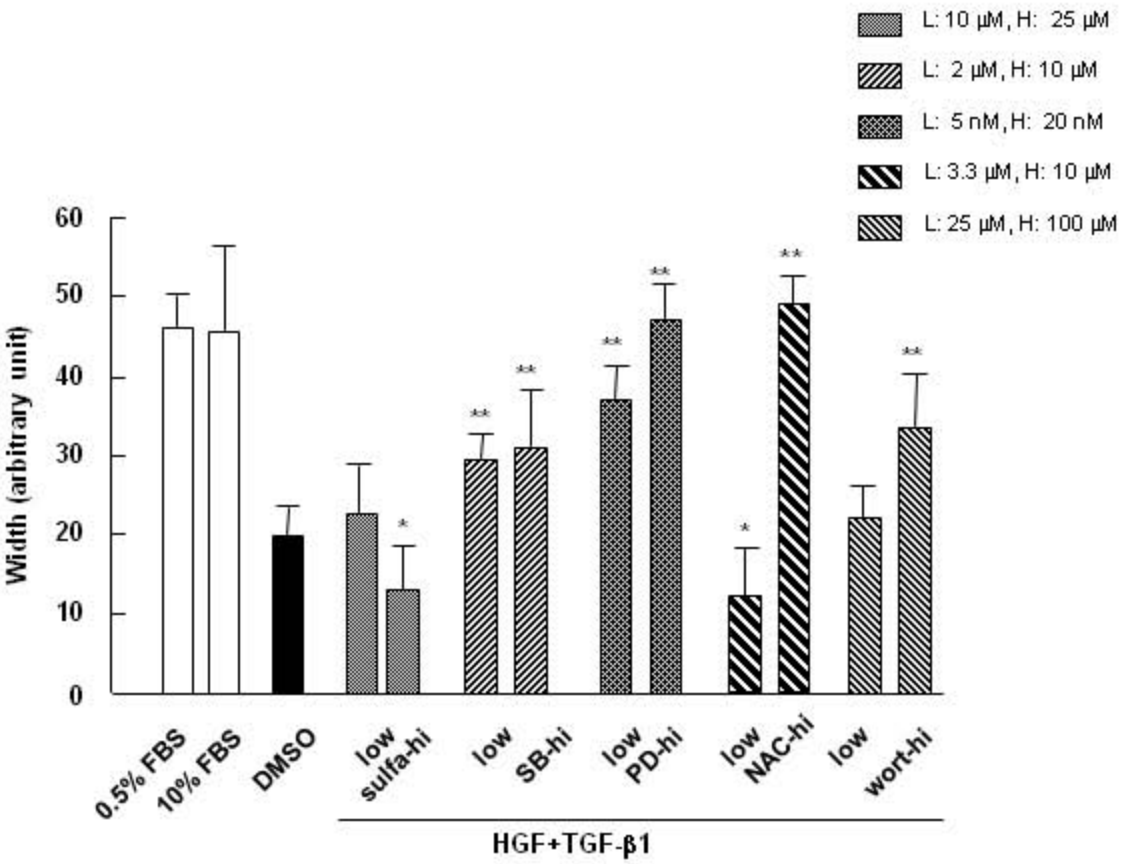
ROS, such as superoxide anions and hydrogen peroxide (H2O2), is produced in mammalian cells in response to the activation of various cell surface receptors, and contribute to intracellular signaling and regulation of various biological activities (Finkel, 2000; Lambeth *et al*., 2000). Receptor-mediated ROS production has been extensively studied in phagocytic cells. The enzyme NADPH oxidase of such cells is composed of at least five protein components, namely two transmembrane flavocytochrome b components (gp91phox and p22phox) and three cytosolic components (p47phox, p67phox, and p40phox) (Babior, 1999). The exposure of resting phagocytic cells to an appropriate stimulus results in extensive phosphorylation of the cytosolic components of NADPH-oxidase and their association with the transmembrane flavocytochrome b components (Ago *et al*., 1999; Cross *et al*., 1999). The assembled oxidase complex catalyzes the transfer of an electron to O2 to yield superoxide anion and H2O2. Several homologs (Nox-1, Nox-3, Nox-4, Nox-5, Duox1, and Duox2) of gp91phox (Nox-2) have been identified in various non-phagocytic cells (Cheng *et al*., 2001). Based on the effect of Nox-1 antisense RNA in smooth muscle cells, Nox-1 has been implicated in PDGF-induced ROS production (Lassegue *et al*., 2001). Nox-4 has also been reported to play a central role in LPS-induced pro-inflammatory responses of endothelial cells in an ROS-dependent manner (Park *et al*., 2006), and is shown to be expressed in melanoma cells as well as normal human epidermal melanocytes (Brar *et al*., 2002). To the best of our

knowledge, this is the first report showing the involvement of Nox-1 and Nox-4 in keratinocyte migration.

Although ROS generation was focused as one of the responsible signaling pathways in keratinocyte migration, it seems that numerous different major signaling pathways are necessary for the completion of this process: (i) Exogenous addition of H2O2 was not sufficient to induce cell migration (Figure 6), (ii) the inhibition of PI3K with two different inhibitors also prevented HaCaT cell migration without affecting ROS level (Figure 5), (iii) several different inhibitors could abolish the HaCaT cell migration (Supplemental Data Figure S1). These data strongly argues that several different downstream signaling pathways are in volved in keratinocyte migration. Another well-documented mechanism of enhanced keratinocyte migration other than ROS generation is the secretion of EGF family ligands such as EGF, TGF-α, amphiregulin, and HB-EGF, which leads to the autocrine activation of EGFR (Coffey *et al*., 1987; Cook *et al*., 1991; Higashiyama *et al*., 1991). Interestingly, Ellis *et al*. (2001) showed that scrape-wounding epithelial cell monolayers induce HB-EGF mRNA expression by a mechanism that most probably require Erk-1 and -2 activation, leading to the migration of the cells via activation of EGFR (Ellis *et al*., 2001). Notably, phosphorylation of both Erk-1 and -2 was significantly increased and sustained by treatment with a combination of both growth factors in our system, and signal inhibitors such as PD98059 and U0126 showed dose-dependent inhibition of HaCaT cell migration without any significant effect on cell proliferation (unpublished observation by Nam). However, we could not observe the activation of EGFR in our system (Supplemental Data Figure S3).

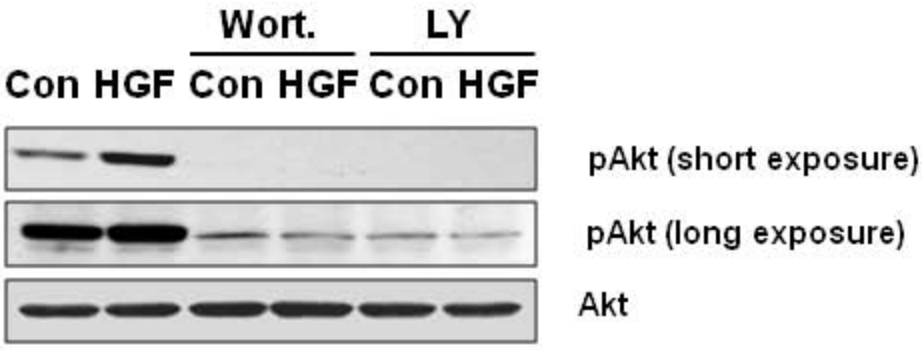
Re-epithelialization by proliferation and migration of keratinocytes from the margin is one of the principal steps in the complex process of wound healing. Thus, enhancement of re-epithelialization would be beneficial in wound healing process. We have shown in the present study that co-treatment of wounded HaCaT cells with HGF and TGF-β1 enhanced keratinocyte migration. Since the use of growth factors as a therapeutic measure to enhance wound healing process appears to be a promising approach, it would be very interesting to test whether combined growth factor treatment would also be beneficial for wound healing *in vivo*.

Supplementary Fig S1.



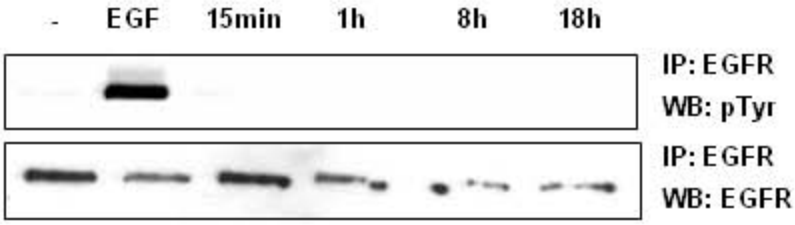
Supplementary Fig S1. Several inhibitors could block the HaCaT cell migration induced by co-treatment with HGF and TGF-β1. HaCaT cells were pre-treated with various cell signaling inhibitors for 30 min, and then were scratched using a micropipette tip, and cytokines were added as indicated with indicated doses of respective inhibitors for 30 h. Cells were stained with crystal violet. Width of the wound was measured by using ocular lens with ruler. Data are presented as mean ± SD of three independent experiments. (* ; $p<0.05$, ** ; $p<0.01$ compared to 0.5% FBS control by Student's t-test). DMSO; vehicle control, sulfa; sulfasalazine, SB; SB203580, PD; PD98059, NAC; N-acetylcysteine, wort; wortmannin inhibitors.

Supplementary Fig S2.



Supplementary Fig S2. Inhibition of cytokine-induced Akt phosphorylation by Wortmannin and LY294002. HaCaT cells grown in 0.5 % serum free media were pretreated for 1 hour without or with 1 μM wortmanin or 30 μM LY294002. Then, cells were treated without or with 30 units/ml of HGF for 15 min, followed by western blotting. Both long and short exposure data are shown.

Supplementary Fig S3.



Supplementary Fig S3. EGFR was not activated by the combined treatment with HGF and TGF-β1. HaCaT cells were incubated in the media containing HGF(30 units/ml) and TGF-β1 (2 ng/ml) for indicated durations. After then, cells were lyses with RIPA buffer containing protease and phosphatase inhibitors followed by immunoprecipitation with anti-EGFR IgG. Western blot analysis for phosphotyrosine as well as EGFR was performed.